ASSOCIATION ANALYSIS BETWEEN HOTAIR RS1899663 SINGLE NUCLEOTIDE POLYMORPHISM AND CLEAR CELL RENAL CELL CARCINOMA DEVELOPMENT IN UKRAINIAN POPULATION

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ABSTRACT
The aim: to study the association between rs1899663-polymorphic variant of HOTAIR gene and clear cell renal cell carcinoma (CCRC) development in Ukrainian population.
Materials and methods: whole venous blood from 101 Ukrainians with CCRC (42 females and 59 males) and 100 control subjects (34 females and 66 males) were enrolled in the study. DNA extraction was performed using GeneJET Whole Blood Genomic DNA Puriﬁcation Mini Kit (Thermo Fisher Scientiﬁc, USA). Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) was used for HOTAIR rs1899663 genotyping. The Statistical Package for Social Science software (SPSS, version 17.0, Chicago, IL, USA) was used for all calculations.
Results: It was found the lack of association between HOTAIR rs1899663 single nucleotide polymorphism and CCRC emergence as well as tumor metastasis property in dominant, recessive, over-dominant and additive crude models of inheritance, as well after the adjustment for age, sex, smoking and excessive alcohol consumption (P > 0.05).
Conclusions: No association was found between HOTAIR rs1899663-polymorphic variant and CCRC development in Ukrainian population. Further studies with extended samples are required to validate these results.

KEY WORDS: clear cell renal cell carcinoma, long non-coding RNA, HOTAIR, gene polymorphism

INTRODUCTION
It has always been believed that mutations are the basis of cancer development. But since the discovery of epigenetics, it has become clear that the combination of genetic and epigenetic changes can lead to cancer [1]. Epigenetics studies changes in gene expression that are not related to variation in DNA sequence [2]. There are some epigenetic modifications described in the literature: DNA methylation, histone acetylation or deacetylation and histone modification [3].

The main part of transcripts is represented by non-coding RNAs (ncRNAs), which divided into two major groups: short and long. Long ncRNAs (lncRNA) have more than 200 nucleotides and are mainly transcribed by RNA polymerase II. They are involved in gene regulation, cell differentiation and development, inactivation of X chromosome etc. One of the representatives of lncRNA is Hox transcript antisense intergenic RNA (HOTAIR), which was discovered by Rinn et al [4].

The HOTAIR is located at 12 chromosome inside the HoxC locus, particular, between HoxC11 and HoxC12 and includes 12 649 nucleotides. The lncRNA HOTAIR turns on 2 158 nucleotides. HOTAIR can act due to its interaction with different complexes, for example Polyclomb repressive complex 2 (PRC2) and Lysine-specific histone demethylase 1 (LSD1), effect on the expression of miRNAs, such as miR-122, miR-17-5p, miR-206, regulate epithelial mesenchymal transition (EMT) etc. [5-8]. Due to PRC2 and LSD1, methylation and demethylation of the corresponding genes are carried out, which leads to a decrease in their expression. For example, these complexes inhibit genes such as tumor and metastasis suppressor genes (RKIP, P5394, Kruppel-like factor 2, RUNX3, SLIT2, DAB2IP etc.) [5, 9-14].

Moreover, it has been found that the overexpression of HOTAIR moderately increases the rate of growth of the primary tumor and promotes the metastasis of cancer. It was observed an overexpression of HOTAIR in renal cell carcinoma cells in comparison with normal renal tissue [15].

Clear cell renal cell carcinoma (CCRC) is the most common subtype of renal cell carcinoma (RCC), which is characterized by accumulation of lipid and glycogen in the cytoplasm of tumor cells that leads to the predominance of clear cell histology [16]. It is the most common and malignant cancer, because of its high metastatic potential, rates of invasiveness and mortality. It accounts for about 65%–75% of all RCCs [17, 18, 19]. There are a lot of risk factors for CCRC, including genetic, epigenetic, and modifiable factors. The main modifiable factors are age, sex, excessive alcohol consumption and cigarette smoking [20].

A single-nucleotide polymorphism (SNP) is the most common type of genetic variation which means the variation in a single nucleotide that has a specific position in the genome. Genes containing SNPs can produce several allelic forms of ncRNAs, which can differ in their effect on gene regulation, chromatin modification and so on [21]. Today, 3 828 single-nucleotide polymorphisms of the HOTAIR
**Table I.** Clinical characteristics of the patients with CCRCC and control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CCRCC (n = 101)</th>
<th>Control (n = 100)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years ± SD</td>
<td>55.3 ± 10.41</td>
<td>77.38 ± 8.49</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>42/59</td>
<td>34/66</td>
<td>0.268</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>49 (48.51)</td>
<td>27 (27)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

CCRCC – clear cell renal cell carcinoma; n – number of cases; P – indicator of statistical significance. Categorical variables were compared by χ²-test, quantitative variables - by t-test.

**Table II.** Clinical characteristics of the patients with and without CCRCC metastasis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>With metastasis (n = 29)</th>
<th>Without metastasis (n = 72)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years ± SD</td>
<td>58.03 ±11.54</td>
<td>54.21±9.78</td>
<td>0.095</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>11/18</td>
<td>31/41</td>
<td>0.636</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>14 (48.3)</td>
<td>35 (48.6)</td>
<td>0.976</td>
</tr>
<tr>
<td>Drinkers, n (%)</td>
<td>18 (62.1)</td>
<td>46 (63.9)</td>
<td>0.854</td>
</tr>
</tbody>
</table>

CCRCC – clear cell renal cell carcinoma; n – number of cases; P – indicator of statistical significance. Categorical variables were compared by χ²-test, quantitative variables - by t-test.

Humans (NCBI) have been studied. The essence of HOTAIR rs1899663-polymorphism is the replacement of guanine by thymine at the 12 chromosome at 53967210th position. It is located in the second intron site in 4903 position (according to NC_000012.12 and NR_003716.3) [22]. Therefore, it was decided to check the association between HOTAIR rs1899663-polymorphism and the development of CCRCC in Ukrainian population.

**THE AIM**

The aim was to study the association between rs1899663-polymorphic variant of HOTAIR and clear cell renal cell carcinoma (CCRCC) development in Ukrainian population.

**MATERIALS AND METHODS**

**STUDY POPULATION**

It was used the whole venous blood of 101 Ukrainians with CCRCC (42 females and 59 males; mean age [±SD] 55.31±10.41) and 100 subjects (34 females and 66 males; mean age 77.38 ± 8.49) as a control (Table I). It was found 29 subjects with metastasis (11 females and 18 males; mean age 58.03±11.54) among oncological patients (Table II).

Each cancer patient was diagnosed from March 2001 to May 2016 with further observation in Sumy Regional Clinical Oncology Dispensary. All oncological patients underwent radical tumor removal with further histological examination. All cancer patients had clinical stage II (TNM Classification of Malignant Tumors). Final morphological diagnosis of CCRCC was estimated according to the European Association of Urology Guidelines. It is worth paying attention to the fact that the mean age of the control group is significantly higher than that in cancer group. Thereby, the risk of cancer development in the control subjects decreases with age that improves the reliability of the control group. The study protocol complied with the Declaration of Helsinki and was approved by the Ethic Committee of the medical Institute of Sumy State University (N9/05.12.11). All individuals gave voluntary informed written consent.

**GENOTYPING**

DNA was isolated from venous blood of 201 subjects using GeneJet Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA). Polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) was used for genotyping HOTAIR rs1899663 SNP. It was used the 2 mM MgSO₄, 0.2 mM dNTPs (Thermo Fisher Scientific, USA), 5 µL 5×PCR buffer, 1U Taq DNA polymerase (Thermo Fisher Scientific, USA), and 75–100 ng DNA for the reaction mixture for PCR (total volume 25 µL). The nucleotide structure of the primers, PCR stages and PCR amplicon size are shown in the Table III. PCR was conducted in Thermocycler GeneAmp PCR System 2700 (Thermo Fisher Scientific, USA).

The primers were chosen to limit the 401 bp DNA sequence, which contains the rs1899663-polymorphic site. However, due to the presence of a constitutive restriction site, the amplicon was split by the BseGI endonuclease into two parts of 76 and 325 bp. An additional BseGI restriction site was formed due to the transversion of G → T in 4093 (rs1899663-polymorphism) position of the HOTAIR (NC_000012.12). At the same time, along with the formation of a fragment of 76 bp, a fragment of 325 bp was cut into two additional ones – 63 and 262 bp. Instead, only two fragments – 76 bp and 325 bp were formed in the presence of the G-allele in the amplicon. Horizontal electrophoresis (10 V/cm) in 2.5% agarose gel with the

**Table III.** PCR conditions for HOTAIR rs1899663-polymorphism.

<table>
<thead>
<tr>
<th>Primers</th>
<th>PCR stages</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>S’GAAAGCCAGGATCATTAAA3’</td>
<td>Denaturation</td>
<td>Hybridization</td>
</tr>
<tr>
<td>94°C – 45 s</td>
<td>59°C – 45 s</td>
<td>72°C – 45 s</td>
</tr>
<tr>
<td>S’GGGCTCAGGAGACCTTAAAG3’</td>
<td>Note: bp: base pairs</td>
<td></td>
</tr>
</tbody>
</table>
addition of a bromide ethidium solution (10 mg/ml) was used to separate the restriction products. Discrimination of HOTAIR rs1899663-polymorphism genotypes was performed by the transilluminator ("Biocom", Russia).

**STATISTICAL ANALYSIS**

The Online Calculator of Hardy-Weinberg equilibrium (https://wpcalc.com/en/equilibrium-hardy-weinberg) was used to determine the distribution of alleles in groups of comparison

**Table VI. Distribution of alleles and genotypes for the HOTAIR rs1899663-polymorphism among patients with and without CCRC metastasis.**

<table>
<thead>
<tr>
<th></th>
<th>With metastasis (n = 29)</th>
<th>Without metastasis (n = 72)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td><strong>Genotypes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>12</td>
<td>41.4</td>
<td>28</td>
</tr>
<tr>
<td>GT</td>
<td>14</td>
<td>48.3</td>
<td>39</td>
</tr>
<tr>
<td>TT</td>
<td>3</td>
<td>10.3</td>
<td>5</td>
</tr>
<tr>
<td><strong>Alleles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>38</td>
<td>65.5</td>
<td>95</td>
</tr>
<tr>
<td>T</td>
<td>20</td>
<td>34.5</td>
<td>49</td>
</tr>
</tbody>
</table>

CCRC – clear cell renal cell carcinoma; n – number of cases; P – indicator of statistical significance.

and Hardy-Weinberg equilibrium (HWE) testing. All computations were made in the Statistical Package for Social Science software (SPSS, version 17.0, Chicago, IL, USA). Chi square (χ²) test (comparing the frequency of alleles and genotypes and other variables) and two-tailed Student's t-test (comparison of averages between two groups) were used in the study. Shapiro-Wilk test confirmed the normal distribution. It was used the logistic regression to estimate the odds ratio (OR) and 95% confidence interval (CI) in the framework of recessive, dominant, over-dominant and additive inheritance models. Multivariable logistic regression was used for smoking, sex, age, and excessive alcohol consumption adjustment. All statistical tests were based on a two-tailed probability; a value of P < 0.05 was accepted as significant.

**RESULTS AND DISCUSSION**

The clinical characteristics of the study groups are shown in Table I. The cancer group was included 101 individuals with CCRC with 55.31 ± 10.41 as an average age. The control group consisted of 100 subjects with an average age of 77.38 ± 8.49, which was much higher. Thus, between cancer and control groups the significant difference was found in average age (P < 0.001) as well, as in smokers (P = 0.002). At the same time, there were no significant differences in sex distribution between these two groups (P = 0.268).

The distribution of alleles and genotypes in case and control groups is presented in Table IV. Alleles distribution
Table VII. Analysis of the association between the HOTAIR rs1899663-polymerorphism and the development of metastasis of CCRC.

<table>
<thead>
<tr>
<th>Model</th>
<th>P</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dominant</td>
<td>0.817</td>
<td>0.902 (0.375-2.169)</td>
<td>0.880</td>
<td>0.933 (0.382-2.279)</td>
</tr>
<tr>
<td>Recessive</td>
<td>0.569</td>
<td>1.546 (0.345-6.938)</td>
<td>0.608</td>
<td>1.489 (0.325-6.829)</td>
</tr>
<tr>
<td>Over-dominant</td>
<td>0.592</td>
<td>0.790 (0.333-1.873)</td>
<td>0.666</td>
<td>0.824 (0.342-1.984)</td>
</tr>
<tr>
<td>Additive (^1)</td>
<td>0.677</td>
<td>1.400 (0.287-6.818)</td>
<td>0.694</td>
<td>1.381 (0.277-6.872)</td>
</tr>
<tr>
<td></td>
<td>0.703</td>
<td>0.838 (0.337-2.083)</td>
<td>0.773</td>
<td>0.872 (0.345-2.203)</td>
</tr>
</tbody>
</table>

CCRC – clear cell renal cell carcinoma; Pc: crude P value; ORc: crude odds ratio; CI: confidence interval; Pc: P value adjusted for age, sex, smoking and excessive alcohol consumption; ORc: adjusted odds ratio. Upper row in the additive model of inheritance – comparison between TT and GG genotypes; lower row – between GT and GG genotypes.

matched with HWE expectation (P_{HWE} = 0.862). Both the distribution of genotypes and alleles had no significant differences (Pc = 0.207; P = 0.189).

The results of analysis of the association between the HOTAIR rs1899663-polymerorphism and the development of CCRC are summarized in Table V. There was no association in all crude models of inheritance as well as after the adjustment for covariates (P > 0.05).

Moreover, 29 subjects with metastasis among oncological patients were found, but there was no significant difference in all parameters: age (P = 0.095), sex (P = 0.636), smokers (P = 0.976), drinkers (P = 0.864) (Table II). Also, there was no association in distribution of alleles and genotypes among patients with and without CCRC metastasis (Pc = 0.790; P = 0.951) (Table VI).

The results of analysis of the association between the HOTAIR rs1899663-polymerorphism and the development of metastasis of CCRC are represented in Table VII. There was no significant difference in all crude regression models. Also, there was no association after the adjustment for sex, age, smoking and excessive alcohol consumption (P > 0.05).

In this study we have checked the association between HOTAIR rs1899663-polymerorphism and the development of CCRC in Ukrainian population. The HOTAIR is located at 12 chromosome inside the HoxC locus and includes 12 649 nucleotides. It was found by John L. Rinn et al. (2007) as a regulator of Chromatin Silencing [4]. They also described in detail the mechanism of HOTAIR’s action through PRC2.

Since that time, many scientists studied the association between HOTAIR’s overexpression and development of different types of cancer. In 2010 Miao-Chih Tsai et al. showed that a 3’ domain of HOTAIR binds the LSD1/CoREST/REST complex [16]. Zh–Yuan Xu et al. (2013) have found that inhibition of HOTAIR reverses EMT in gastric cancer [17]. Xiao Song Ge et al. (2013) showed that HOTAIR inhibits Wnt inhibitory factor 1 expression and activates Wnt pathway. The Wnt/b-catenin signaling pathway plays an important role in migration and cell proliferation and cancer progression [18]. Liu XH et al. (2014) showed that HOTAIR can act as a competing endogenous RNA. Through miR-331-3p it can regulate HER2 expression in gastric cancer [19]. Mohammadreza Hajjari and Abbas Salavaty (2015) showed the role of HOTAIR’s overexpression, based on information from various articles. Studies have shown a positive link between the HOTAIR’s overexpression and cancer progression and metastasis. Further, they gathered together all the factors that may affect on HOTAIR’s expression. For example, Ago2 complex can suppress the function of HOTAIR unlike osteopontin, which causes its overexpression [5].

This IncRNA has a lot of different polymorphisms, so we have chosen one of them. The essence of HOTAIR rs1899663-polymerorphism is the replacement of guanine by thymine at the 12 chromosome at 53967210th position [22]. It is located in the intronic region of HOTAIR [23]. There are three major HOTAIR transcript variants that have an appropriate SNP localization: variant 1 – 325+59G>T (NR_047517.1); variant 2 – 266+59G>T (NR_003716.3); variant 3 – 299-833G>T (NR_047518.1).

During the study we discovered the following T-allele distribution for Ukrainian population: 0.358 for cancer group and 0.405 for control group. There were no significant differences in distribution of alleles in case and control groups. The T-allele distribution in non-metastasis and metastasis groups was 0.34 and 0.345 respectively. Also, there were no significant differences in distribution of alleles in these two groups.

The 1000 Genomes Project showed the minor allele frequency in 5 populations: East Asian (EAS) – 0.205, South Asian (SAS) – 0.31, European (EUR) – 0.268, African (AFR) – 0.179 and American (AMR) – 0.36. The most similar results for Ukrainian control group were obtained only for American (0.36). So, there are ethnic differences in the rs1899663 allele’s distribution [22].

The number of works devoted to this polymorphism was significantly increased over the past few years. Xu Yang et al. (2018) have found a significant association between rs1899663 C>A (P = 0.29) polymerism and increased neuroblastoma risk [24]. At the same time, most authors such as Shun-Long Weng et al. (2018), Min-Che Tung et al. (2019), Xu T et al. (2019) etc. have not found a significant association between rs1899663 polymerism and cancer development [25–27].

At this study we investigated only the association between the HOTAIR rs1899663-polymerism and CCRC development. It is of interest to study the HOTAIR expression levels depending on the allelic variant.
CONCLUSIONS
In this work it was studied the involvement of rs1899663-poly-
morphism in the development of CCRCC. The alleles and
and genotypes distribution of rs1899663-polyorphism for
Ukrainian population was investigated. It was found no
significant differences between HOTAIR, rs1899663-poly-
morphism and CCRCC as in most works of other authors. It
can be concluded that there are more significant predictors.

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Conflict of interest:
The Authors declare no conflict of interest

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D — Writing the article, E — Critical review, F — Final approval of the article